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Pharmacological characterization of rebamipide: its cholecystokinin CCK_1 receptor binding profile and effects on Ca^{2+} mobilization and amylase release in rat pancreatic acinar cells

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Abstract

We previously reported that rebamipide (2-(4-chlorobenzoylamino)-3-[2(1*H*)-quinolinon-4-yl]-propionic acid) generated oscillations of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) probably through the activation of cholecystokinin type 1 (CCK_1) receptors in rat pancreatic acinar cells. Therefore, in the present study, we aimed to establish the pharmacological characteristics of rebamipide in rat pancreatic acinar cells. CCK-8S and rebamipide inhibited [^{125}I]BH-CCK-8S binding to rat pancreatic acinar cell membranes with IC_{50} values of 3.13 nM and 37.7 μM, respectively. CCK-8S usually evoked $[Ca^{2+}]_i$ oscillations at concentrations lower than 50 pM, and it induced biphasic $[Ca^{2+}]_i$ increases at higher concentrations. In contrast to CCK-8S, rebamipide only induced $[Ca^{2+}]_i$ oscillations at all the concentrations we used in this study. In addition, rebamipide was shown to inhibit high concentrations of CCK-8S-induced biphasic increases in $[Ca^{2+}]_i$, suggesting that rebamipide might be a partial agonist at cholecystokinin CCK_1 receptors. Although rebamipide induced $[Ca^{2+}]_i$ oscillations by activating the cholecystokinin CCK_1 receptors, rebamipide did not cause amylase release and only inhibited CCK-stimulated amylase release reversibly and dose-dependently. However, rebamipide did not inhibit carbachol-, vasoactive intestinal polypeptide (VIP)-, and forskolin-induced amylase releases. These data indicate that rebamipide functions as a partial agonist for Ca^{2+}-mobilizing action, and it is also an antagonist for the amylase-releasing action of CCK.

Keywords: Rebamipide; Cholecystokinin CCK_1 receptor; $[Ca^{2+}]_i$ oscillation; Amylase

1. Introduction

Rebamipide (2-(4-chlorobenzoylamino)-3-[2(1*H*)-quinolinon-4-yl]-propionic acid) is an antiulcer agent, which exerts its cytoprotective effect by increasing prostaglandin generation in the gastric mucosa and by scavenging reactive oxygen species (Yamasaki et al., 1987; Ogino et al., 1992; Iinuma et al., 1998; Haruma and Ito, 2003). In addition to this protective effect on gastric mucosa, rebamipide was also shown to have a preventive effect on oxidative stress-induced acute pancreatitis (Seo et al., 2002).

However, apart from its effects on the generation of prostaglandin and reactive oxygen species, rebamipide was previously demonstrated to evoke oscillations of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in rat pancreatic acinar cells in the concentration range 5–500 μM (Moon et al., 2000). Since the $[Ca^{2+}]_i$ oscillations were inhibited by the specific cholecystokinin type 1 (CCK_1) receptor antagonist L-364,718 (devazepide), but not by atropine or the cholecystokinin CCK_2 receptor antagonist (3*R*)-(+)-*N*-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1*H*-1,4-benzodiazepin-3-yl)-3-methylphenylurea (L-365,260), we suggested that rebamipide-evoked $[Ca^{2+}]_i$ oscillations were due to the activation of cholecystokinin CCK_1 receptors in rat pancreatic acinar cells.

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The signal transduction pathways linked to cholecystokinin CCK_1 receptor activation have been extensively studied in pancreatic acinar cells (Williams, 2001). Stimulation of the cholecystokinin CCK_1 receptors, which are coupled to Gq/11 proteins, increases the production of inositol 1,4,5-trisphosphate (IP_3) through the activation of phospholipase C. IP_3 increases $[Ca^{2+}]_i$ both by mobilization of Ca^{2+} from intracellular Ca^{2+} stores and by stimulation of Ca^{2+} entry across the plasma membrane. Recently, the existence of other Ca^{2+}-mobilizing second messengers, such as cyclic ADP ribose and nicotinic acid adenine dinucleotide phosphate (NAADP), was also reported to be involved in the CCK-induced Ca^{2+} mobilization (Cancela et al., 2000). An increase in $[Ca^{2+}]_i$ triggers exocytosis of secretory granules containing digestive enzymes (Williams, 2001).

Since rebamipide may have a clinically relevant effect on the physiology of pancreas via cholecystokinin CCK_1 receptors, it must be of particular interest to investigate the pharmacological actions of rebamipide at the cholecystokinin CCK_1 receptors and on the cholecystokinin CCK_1 receptor-mediated cell functions in rat pancreatic acinar cells. In the present study, we have found that rebamipide binds saturably to the cholecystokinin CCK_1 receptor and evokes $[Ca^{2+}]_i$ oscillations, although it induces little increase in amylase release.

2. Materials and methods

2.1. Preparation of rat pancreatic acinar cells

Animal use was approved by the Institutional Animal Care and Use Committee of Yonsei University Medical Center. Male Sprague–Dawley rats (150–250 g) were anesthesized with diethyl ether and killed by decapitation. The pancreata were immediately removed and trimmed of fat on ice. The acini were prepared using 50 U/ml collagenase by a slight modification of the methods described previously (Matozaki et al., 1990; Toescu et al., 1993). The isolated pancreatic acinar cells were suspended in a HEPES-buffered physiological solution containing (in mM): NaCl 104, KCl 4.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 0.5, HEPES–Na 25, HEPES-free acid 15, D-glucose 15, minimal Eagle's medium (MEM) amino acids (×1), 1% bovine serum albumin, soybean trypsin inhibitor (4 mg/ml), and L-glutamine 2 (adjusted to pH 7.4, gassed with 100% O_2).

2.2. Cholecystokinin CCK_1 receptor binding study

The binding study of [^{125}I]BH-CCK-8S to its receptor was performed according to the protocol described previously (Sankaran et al., 1980). In brief, the acini were resuspended in the HEPES-buffered physiological solution, and 1-ml aliquots were distributed into 10-ml polystyrole vials. A total of 10 pM [^{125}I]BH-CCK-8S plus various concentrations of unlabeled CCK-8S or rebamipide were added to each vial, and the incubation was carried out for 120 min at 37 °C with shaking (60 cycles/min). After the incubation, the cell suspension was centrifuged at 500×*g* for 3 min, and the obtained pellet was washed twice with 1 ml of 0.9% saline at 4 °C. The radioactivity of the pellet was counted by a gamma scintillation counter and the total radioactivity was determined by counting the total incubation mixture. Nonspecific binding was determined by incubating the acini with labelled CCK in the presence of excess CCK-8S (1 μM). The competitive inhibition of [^{125}I]BH-CCK-8S binding by unlabeled agents was analysed by the GraphPad Prism Program (GraphPad Software, San Diego, CA).

2.3. Amylase assay

Isolated pancreatic acini were centrifuged and then resuspended in fresh HEPES-buffered physiological solution containing 1.5 mM $CaCl_2$. One-milliliter aliquots of acinar suspension distributed into 10-ml polycarbonate Erlenmeyer flasks were incubated with secretagogues at 37 °C for 30 min under constant shaking at 60× per minute, and the incubation was then terminated with centrifugation at 500×*g* for 3 min at 4 °C. The supernatant was reserved on ice for a subsequent amylase assay, which was carried out by a modified method of Bernfeld (1955) using starch as a substrate. The amylase release was expressed as the percentage of the total content of amylase in each sample.

2.4. Fura-2 loading and $[Ca^{2+}]_i$ measurements

The cells were loaded with fura-2 by incubation with 2 μM acetoxymethyl ester of fura-2 (fura-2/AM) in a HEPES-buffered solution equilibrated with 100% O_2 for 40 min at room temperature. They were washed twice and resuspended in a HCO_3^--buffered solution containing (in mM): NaCl 110, KCl 4.5, NaH_2PO_4 1.0, $MgSO_4$ 1.0, $CaCl_2$ 1.5, $NaHCO_3$ 25, HEPES–Na 5, HEPES-free acid 5, D-glucose 10, and equilibrated with 95% O_2 and 5% CO_2 to give a pH of 7.4. The cells were allowed to attach to a coverslip that formed the base of a cell chamber mounted on the stage of an inverted microscope and were superfused with the HCO_3^--buffered solution at a flow rate of 2 ml/min. The buffer was continuously gassed with 5% CO_2 in O_2 during the experiments, and a water-jacketed perfusion line between the pump and the cell chamber was maintained the temperature of the perfusate at 37 °C. $[Ca^{2+}]_i$ was measured by spectrofluorometry (Photon Technology International, Brunswick, NJ) with excitations at 340 and 380 nm, and emission was measured at 510 nm. The values of $[Ca^{2+}]_i$ were calculated from the ratio of fluorescence intensities ($F_{340/380}$) according to Grynkiewicz et al. (1985).

2.5. Materials

Collagenase (type IV), soybean trypsin inhibitor, HEPES, bovine serum albumin, MEM amino acids,

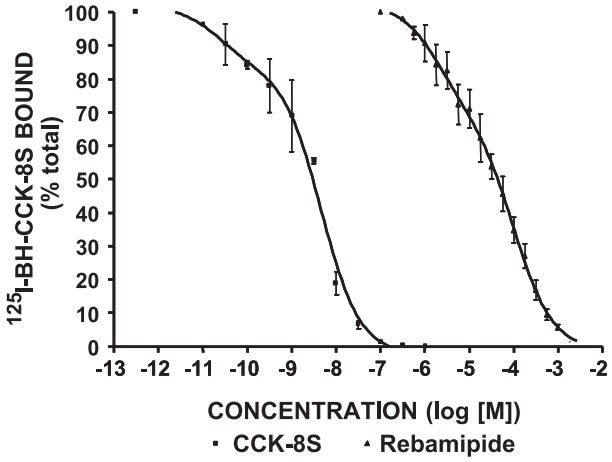

Fig. 1. Competition of CCK-8S and rebamipide for binding of [^{125}I]CCK-8S to rat pancreatic acinar cells. Cells were incubated with 10 pM [^{125}I]CCK-8S in the presence or absence of increasing concentrations of unlabeled CCK-8S or rebamipide for 120 min. Binding data are expressed as percentages of maximal binding, which was observed in the absence of competitor. Values are mean±S.E.M. from three separate experiments for CCK-8S and five for rebamipide.

glutamine, carbachol, vasoactive intestinal polypeptide (VIP), forskolin, and CCK-8S were purchased from Sigma (St. Louis, MO). [^{125}I]BH-CCK-8S was from Amersham (Piscataway, NJ).

Fura-2/AM was obtained from Molecular Probes (Eugene, OR). Rebamipide was supplied from Korea Otsuka Pharmaceutical (Seoul, Korea) and was directly dissolved in solutions just before each experiment was performed.

2.6. Data analysis

The results presented are mean±S.E.M. Statistical analysis was performed by unpaired Student's *t* test. *P* values lower than 0.05 were considered to be statistically significant.

3. Results

3.1. Binding of CCK-8S and rebamipide to rat pancreatic cholecystokinin CCK_1 receptors

The ability of rebamipide to occupy the cholecystokinin CCK_1 receptors on pancreatic acinar cells was assessed by the displacement of [^{125}I]BH-CCK-8S. As shown in Fig. 1, unlabeled CCK-8S and rebamipide inhibited the binding of [^{125}I]BH-CCK-8S with IC_{50} of 3.13±0.83 nM (n=3) and 37.7±3.4 μM (n=5), respectively. To assess the affinities of rebamipide and CCK-8S for each pancreatic cholecystokinin CCK_1 receptor binding site, the dose inhibition curves for rebamipide and CCK-8S against [^{125}I]BH-CCK-8S binding were analysed using the GraphPad Prism Program. As reported previously (Gaisano et al., 1989), the inhibition of [^{125}I]BH-CCK-8S binding to cholecystokinin CCK_1 receptor by CCK-8S was best fitted by a two-binding site model; the K_d values are 22.9±6.0 pM for the high-affinity site and 4.65±0.54 nM for the low-affinity site. In contrast to CCK-8S, rebamipide was not shown to discriminate between the two binding sites, although it completely displaced [^{125}I]BH-CCK-8S at both affinity sites (K_d value; 39.5±3.5 μM).

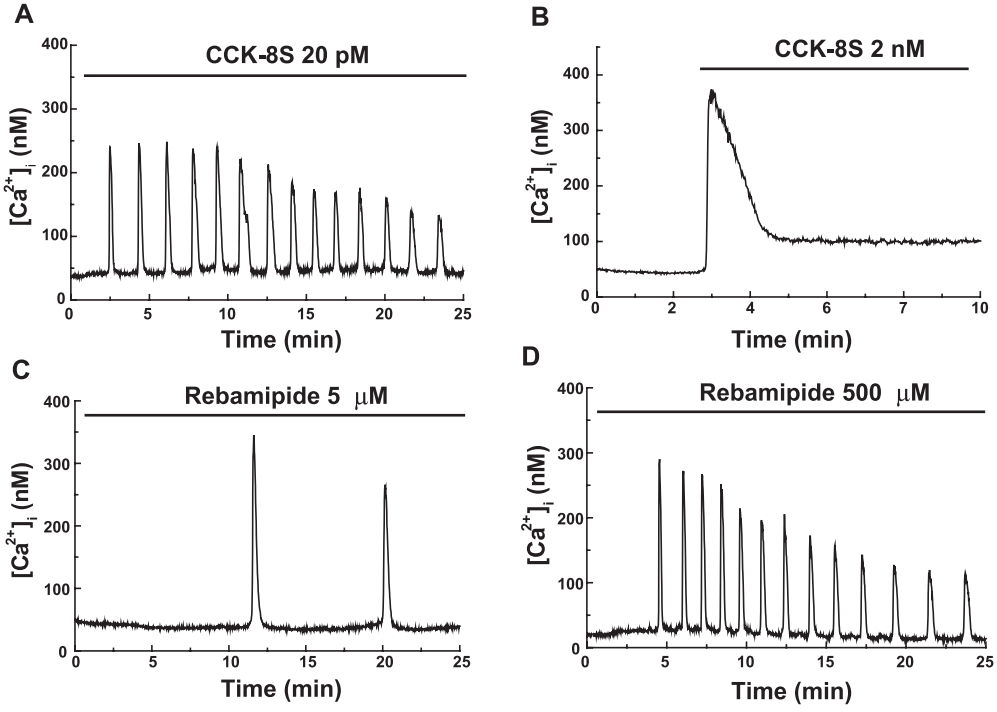

Fig. 2. Typical Ca^{2+} responses stimulated with noted concentrations of CCK-8S and rebamipide in rat pancreatic acinar cells. Each panel is representative of four to six separate experiments.

3.2. Effect of CCK-8S and rebamipide on changes in $[Ca^{2+}]_i$

CCK has been shown to induce $[Ca^{2+}]_i$ oscillations at physiological concentrations, whereas it induced biphasic $[Ca^{2+}]_i$ increases at high concentrations. In our system, CCK-8S at concentrations lower than 50 pM usually evoked $[Ca^{2+}]_i$ oscillations, whereas it induced biphasic $[Ca^{2+}]_i$ increases at higher concentrations. However, in contrast to CCK-8S, rebamipide only induced $[Ca^{2+}]_i$ oscillations at all concentrations we used in this study. Fig 2 shows the typical changes in $[Ca^{2+}]_i$ in response to CCK-8S (20 pM and 2 nM) and rebamipide (5 and 500 μM), respectively. Interestingly, when the cells were pretreated with 300 μM rebamipide, the 2 nM CCK-8S-induced biphasic increase in $[Ca^{2+}]_i$ was abolished (Fig. 3), suggesting that rebamipide is a partial agonist of cholecystokinin CCK_1 receptors.

3.3. Effect of rebamipide on amylase release

CCK has been known to produce a typical biphasic dose–response curve for amylase release (Stark et al., 1989). In this study, we also observed that the amylase release increased as CCK-8S concentration increased, reaching a maximum at 100 pM, and then decreased. In contrast to this, rebamipide did not significantly increase the amylase release (Fig. 4A). Furthermore, 100–1000 μM rebamipide obviously inhibited CCK-8S-induced amylase release and shifted the dose–response curve to the right without altering

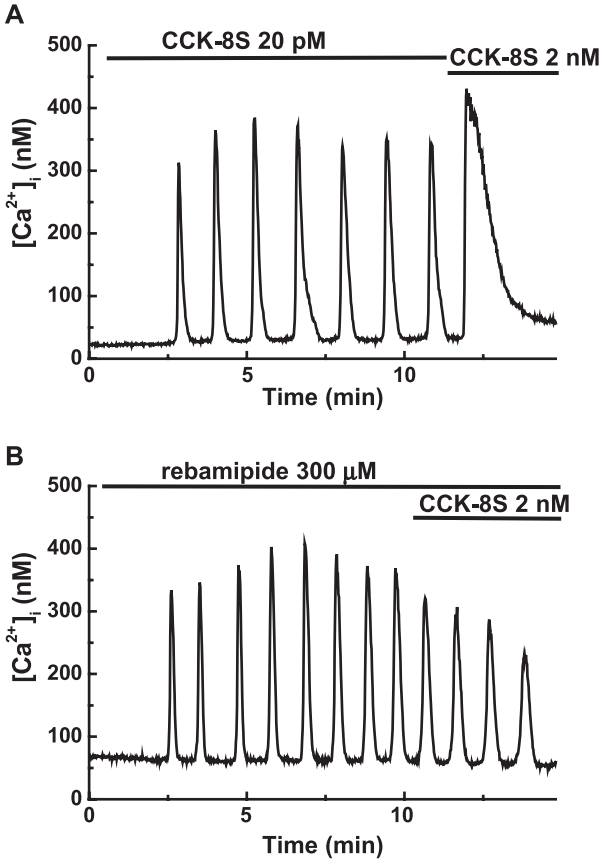

Fig. 3. Inhibitory effect of rebamipide on 2 nM CCK-8S-stimulated biphasic increase in $[Ca^{2+}]_i$ in rat pancreatic acinar cells. Each panel is representative of three to four separate experiments.

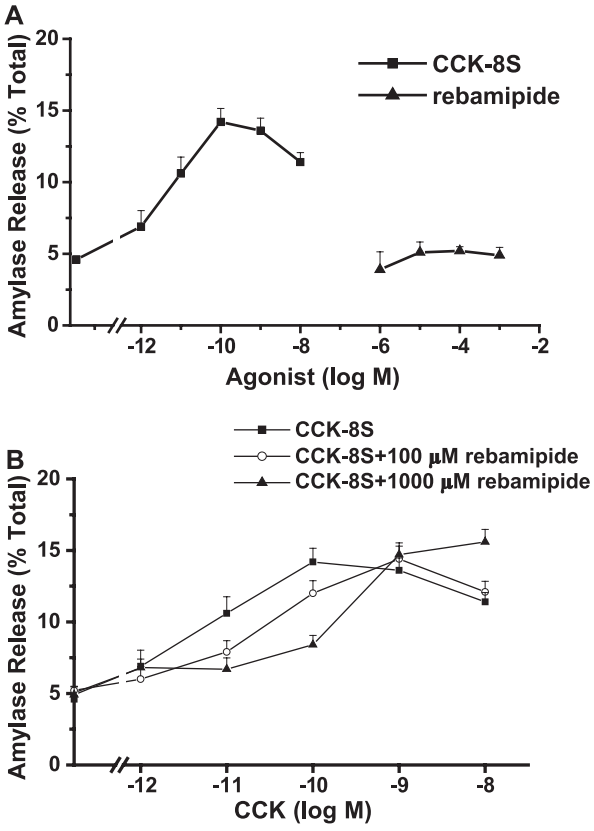

Fig. 4. Dose–response curve for amylase release from rat pancreatic acinar cells in response to CCK-8S and rebamipide. (A) CCK-8S induced a biphasic increase in amylase release while rebamipide did not significantly increase the amylase release. (B) The dose–response curve of CCK-induced amylase releases was shifted to the right by 100 and 1000 μM rebamipide, dose-dependently. Values are mean±S.E.M. from three to six separate experiments.

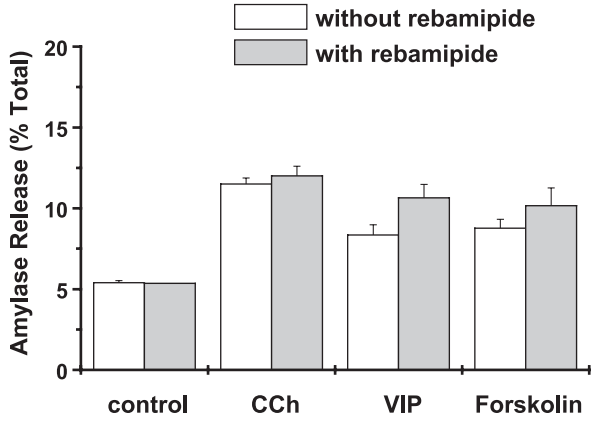

Fig. 5. Effect of rebamipide on carbachol-, VIP-, and forskolin-induced amylase releases. Amylase releases induced by these agents were not inhibited by 1000 μM rebamipide. Values are mean±S.E.M. from three to five separate experiments.

the maximal increase (Fig. 4B). This suggests that rebamipide is a competitive inhibitor of the amylase-releasing action of CCK-8S. To confirm whether the antagonistic effect of rebamipide on amylase release was specific for CCK, we tested if carbachol-, VIP-, and forskolin-induced amylase releases were affected by rebamipide. As shown in Fig. 5, rebamipide did not inhibit the amylase releases induced by these agents.

4. Discussion

In the present study, we have shown that rebamipide produced a concentration-dependent inhibition of [^{125}I]BH-CCK-8S binding to cholecystokinin CCK_1 receptors in rat pancreatic acinar cells in the micromolar range. Since we already reported that rebamipide induced $[Ca^{2+}]_i$ oscillations in the concentration range of 5–500 μM, and the $[Ca^{2+}]_i$ oscillations were inhibited by the specific cholecystokinin CCK_1 receptor antagonist L-364,718 but not by atropine or the cholecystokinin CCK_2 receptor antagonist L-365,260 (Moon et al., 2000), these results imply that rebamipide functions as an agonist at the cholecystokinin CCK_1 receptors.

Activation of the cholecystokinin CCK_1 receptors with CCK receptor full agonists, such as CCK-8S, is known to induce two different patterns of Ca^{2+} responses; low concentrations of CCK-8S (<50 pM) generally evoke $[Ca^{2+}]_i$ oscillations, whereas high concentrations of CCK-8S cause biphasic increases in $[Ca^{2+}]_i$, consisting of a rapid increase in $[Ca^{2+}]_i$ followed by a slow decrease to a sustained elevated level (Matozaki et al., 1990). However, the pattern of Ca^{2+} responses stimulated by rebamipide differed from the response to CCK-8S. Rebamipide only evoked $[Ca^{2+}]_i$ oscillations and it was unable to elicit biphasic $[Ca^{2+}]_i$ increases even at high concentrations. In addition to this, rebamipide appeared to antagonize the high concentration of CCK-8S-induced biphasic $[Ca^{2+}]_i$ increases. These results rather suggest that rebamipide is a partial agonist at rat pancreatic cholecystokinin CCK_1 receptors.

Rebamipide is not unique in this respect. The analogues of CCK, such as CCK-JMV-180 and CCK-OPE, only evoke $[Ca^{2+}]_i$ oscillations without inducing biphasic increases in $[Ca^{2+}]_i$ in rat pancreatic acinar cells (Matozaki et al., 1990; Gaisano et al., 1994). The rat pancreatic acinar cells are known to possess two different receptor states: high-affinity receptors and low-affinity receptors (Williams and Blevins, 1993). The high-affinity cholecystokinin CCK_1 receptors have been known to elicit $[Ca^{2+}]_i$ oscillations, while the low-affinity cholecystokinin CCK_1 receptors are responsible for the biphasic increase in $[Ca^{2+}]_i$. CCK-JMV-180 and CCK-OPE are known to activate only the high-affinity cholecystokinin CCK_1 receptors, whereas CCK-8S activates both high- and low-affinity cholecystokinin CCK_1 receptors. Therefore, our findings that rebamipide only induces $[Ca^{2+}]_i$ oscillations and inhibits high concentrations of CCK-8S-evoked biphasic increases in $[Ca^{2+}]_i$ suggest that rebamipide might activate the high-affinity cholecystokinin CCK_1 receptors and inhibit the low-affinity cholecystokinin CCK_1 receptors.

The profile of amylase release in response to rebamipide also differed from that in response to CCK, which has been shown to produce a typical biphasic dose–response curve for amylase release (Stark et al., 1989). In the present study, we also observed that CCK-8S increased amylase release in a dose-dependent manner and concentrations above 1 nM caused a marked reduction in amylase release. This is because the activation of the high-affinity cholecystokinin CCK_1 receptors enhances amylase release, whereas the low-affinity cholecystokinin CCK_1 receptor causes the inhibition of amylase release. However, rebamipide induced little increase in amylase release. This is unexpected because $[Ca^{2+}]_i$ oscillations induced by various agonists, such as acetylcholine, CCK, CCK-JMV-180, and CCK-OPE, were shown to increase the amylase release in rat pancreatic acinar cells. Although the mechanisms by which Ca^{2+} regulates exocytosis in pancreatic acinar cells are poorly understood, Ca^{2+} may directly or indirectly activate the downstream target proteins, such as soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) and Rab proteins, which are involved in the regulation of exocytosis of the secretory granules (Williams, 2001). Therefore, it is possible that rebamipide may somehow inhibit such proteins. Another possible interpretation is that such agonists as acetylcholine, CCK-8S, CCK-JMV-180, and CCK-OPE may activate another intracellular signaling mechanism in addition to $[Ca^{2+}]_i$ oscillations and that the two together are necessary for amylase release. In this case, $[Ca^{2+}]_i$ oscillations per se may not be sufficient to evoke amylase release. Previously, there was a supportive report that $[Ca^{2+}]_i$ oscillations evoked by endothelin induce little increase in amylase release in pancreatic acinar cells (Yule et al., 1992). However, we have so far failed to provide evidences to explain the lack of stimulating effect of rebamipide on amylase release.

Since rebamipide binds saturably to the cholecystokinin CCK_1 receptor of rat pancreatic acinar cells and has little effect on amylase release, rebamipide may antagonize the amylase-releasing action of CCK. In support of this, rebamipide was shown to shift the dose–response curve of CCK-8S-induced amylase release to the right. The amylase releases induced by other stimulators, such as carbachol, VIP, and forskolin, were not inhibited by rebamipide, suggesting that the inhibitory effect of rebamipide on CCK-8S-induced amylase release was due to the competitive binding of rebamipide on the cholecystokinin CCK_1 receptors. Therefore, our study suggests that rebamipide functions as a partial agonist of cholecystokinin CCK_1 receptor-mediated Ca^{2+} mobilizing action, but it antagonizes the cholecystokinin CCK_1 receptor-mediated amylase-releasing action.

Previously, rebamipide was reported to have a protective effect on the generation of an experimental pancreatitis probably due to its antioxidant effect (Seo et al., 2002). Pancreatitis can be induced in many conditions, but the sustained elevation of $[Ca^{2+}]_i$ has been suggested to play an essential role in the pathogenesis of acute pancreatitis (Ward et al., 1995). The increase in $[Ca^{2+}]_i$ may lead to the activation of intracellular proteases resulting in autodigestion (Raraty et al., 2000). Many factors including circulating bile acid (Kim et al., 2002), generation of oxygen free radicals (Schulz et al., 1999) and hyperstimulation with CCK (Raraty et al., 2000) are known to induce $[Ca^{2+}]_i$ increase and cause pancreatitis. Although hyperstimulation of pancreatic acinar cells with CCK does not seem to happen in a normal physiological condition, circulating CCK may be increased during obstructive pancreatitis due to the feedback regulation. In this instance, elevated CCK may exert continuous stimulation on the pancreatic cells and aggravate the pancreatitis. Therefore, rebamipide may have a beneficial effect against acute pancreatitis by antagonizing the high concentration of CCK-induced sustained $[Ca^{2+}]_i$ increase. In support of this, a cholecystokinin CCK_1 receptor antagonist, loxiglumide, was shown to have therapeutic effects on human acute pancreatitis (Ochi et al., 1999). In addition, because the cholecystokinin CCK_1 receptors are involved in various physiological functions, such as gallbladder contraction (Shaffer, 2000), regulation of gastric emptying (Herranz, 2003), and induction of satiety (Woods, 2004), further investigation will be needed to search for other therapeutic potentials.

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